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(54) Title: GENES ENCODING EPSILON LYCOPENE CYCLASE AND METHOD FOR PRODUCING BICYCLIC EPSILON CAROTENE

(57) Abstract

The present invention relates to the DNA sequence for eukaryotic genes encoding ϵ cyclase isolated from romaine lettuce as well as vectors containing the same and hosts transformed with said vectors. The present invention provides methods for controlling the ratio of various carotenoids in a host and to the production of novel carotenoid pigments. The present invention also provides a method for treating disease by administering carotenoids obtained from transformed hosts, or by administering a composition containing the transformed hosts.

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Genes Encoding Epsilon Lycopene Cyclase and Method for Producing Bicyclic Epsilon Carotene

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention describes the DNA sequence for eukaryotic genes encoding ϵ lycopene cyclase as well as vectors containing the same and hosts transformed with these vectors. The present invention also provides a method for augmenting the accumulation of carotenoids and production of novel and rare carotenoids. The present invention provides methods for controlling the ratio of various carotenoids in a host. Additionally, the present invention provides a method for screening for eukaryotic genes encoding enzymes of carotenoid biosynthesis and metabolism. The invention also provides transgenic plants having therapeutic properties, methods for preparing a therapeutic composition, and methods for treating disease by administering the therapeutic plants and compositions.

Discussion of the Background

Carotenoid pigments with cyclic endgroups are essential components of the photosynthetic apparatus in oxygenic photosynthetic organisms (e.g., cyanobacteria, algae and plants; Goodwin, 1980). The symmetrical bicyclic yellow carotenoid pigment β-carotene (or, in rare cases, the asymmetrical bicyclic α-carotene) is intimately associated with the photosynthetic reaction centers and plays a vital role in protecting against potentially lethal photooxidative damage (Koyama, 1991). β-carotene and other carotenoids derived from it or from α-carotene also serve as light-harvesting pigments (Siefermann-Harms, 1987), are involved in the thermal dissipation of excess light energy captured by the light-harvesting antenna (Demmig-Adams & Adams, 1992), provide substrate for the biosynthesis of the plant growth regulator abscisic acid (Rock & Zeevaart, 1991; Parry & Horgan, 1991), and are precursors of vitamin A in human and animal diets (Krinsky, 1987). Plants also exploit carotenoids as coloring agents in flowers and fruits to attract pollinators and agents of seed dispersal (Goodwin, 1980). The color provided by carotenoids is also of agronomic value in a number of important crops. Carotenoids are currently harvested from plants for use as pigments in food and feed.

Two types of cyclic endgroups are commonly found in higher plant carotenoids, these are referred to as the β and ϵ cyclic endgroups (Fig. 2; the acyclic endgroup is referred to as the Ψ or psi endgroup). These cyclic endgroups differ only in the position of the double bond in the ring. Carotenoids with two β rings are ubiquitous, and those with one β and one ϵ ring are common, but carotenoids with two ϵ rings are found in significant amounts in relatively few plants. β-Carotene (Fig. 1) has two β endgroups and is a symmetrical compound that is the precursor of a number of other important plant carotenoids such as zeaxanthin and violaxanthin (Fig. 1).

Carotenoid enzymes have previously been isolated from a variety of sources including bacteria (Armstrong et al., 1989, Mol. Gen. Genet. 216, 254-268; Misawa et al., 1990, J. Bacteriol., 172, 6704-12), fungi (Schmidhauser et al., 1990, Mol. Cell. Biol. 10, 5064-70), cyanobacteria (Chamovitz et al., 1990, Z. Naturforsch, 45c, 482-86) and higher plants (Bartley et al., Proc. Natl. Acad. Sci USA 88, 6532-36; Martinez-Ferez & Vioque, 1992, Plant Mol. Biol. 18, 981-83). Many of the isolated enzymes show a great diversity in function and inhibitory properties between sources. For example, phytoene desaturases from *Synechococcus* and higher plants carry out a two-step desaturation to yield ζ-carotene as a reaction product; whereas the same enzyme from *Erwinia* introduces four double bonds forming lycopene. Similarity of the amino acid sequences are very low for bacterial versus plant enzymes. Therefore, even with a gene in hand from one source, it is difficult to screen for a gene with similar function in another source. In particular, the sequence similarity between bacterial/fungal and cyanobacterial/plants genes is quite low.

The difficulties in isolating related genes is exemplified by recent efforts to isolated the enzyme which catalyzes the formation of β-carotene from the acyclic precursor lycopene. Although this enzyme had been isolated in a bacterium, prior to the invention described in U.S. Serial No. 08/142,195(which is hereby incorporated by reference in its entirety),it had not been isolated from any photosynthetic organism nor had the corresponding genes been identified and sequenced or the cofactor requirements established. The isolation and characterization of the enzyme catalyzing formation of β-carotene in the cyanobacterium *Synechococcus* PCC7942 was described by Cunningham et al. in 1993 and 1994.

The β-cyclase of *Arabidopsis* adds two rings to the symmetrical lycopene to form the

bicyclic β-carotene, but the related ϵ-cyclase of Arabidopsis, which has 36% identity for the predicted amino acid sequences) adds only a single ring to form the monocyclic δ-carotene (Cunningham et al, 1996, Plant Cell 8:1613-1626; U.S. Application No. 08/624,125 filed March 29, 1996, which is incorporated by reference herein in its entirety). These differences in function provide a simple mechanism for adjusting the proportions of β,β-and β,ϵ-carotenoids while at the same time preventing formation of carotenoids with two epsilon rings.

In view of the afore-mentioned deficiencies with prior art methods of producing carotenoids with two epsilon rings, it is clear that there exists a need in the art for such methods.

SUMMARY OF THE INVENTION

Accordingly, a first object of this invention is to provide isolated eukaryotic genes which encode enzymes which encode lycopene epsilon cyclases which form bicyclic epsilon-carotene.

A second object of the present invention is to provide vectors containing said genes.

A third object of the present invention is to provide hosts transformed with said vectors.

A further object is to provide a method for producing a lycopene epsilon cyclase using the transformed host.

A still further object is to provide the lycopene epsilon cyclase so produced.

Another object of the present invention is to provide hosts which accumulates novel or rare carotenoids or which overexpress known carotenoids.

Yet another object of the invention is to provide a method for producing novel or rare carotenoids.

Another object of this invention is to secure the expression of eukaryotic carotenoid-related genes in a recombinant prokaryotic host.

An additional object of the invention is a method of preparing a therapeutic composition comprising either the host cell which expresses the lycopene epsilon cyclase or the isolated carotenoids produced by the host cell containing the lycopene epsilon cyclase.

Another object of the invention is to provide a method for the treatment of disease by

providing to a patient in need thereof, an amount of the rare carotenoids in an amount sufficient to treat the disease.

These and other objects of the present invention have been realized by the present inventors as described below.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

Figure 1 depicts possible routes of synthesis of cyclic carotenoids and some common plant and algal xanthophylls (oxycarotenolds) from lycopene. Activities of the ϵ- cyclase enzyme of lettuce are indicated by bold arrows labelled with ϵ. The reaction leading to ϵ-carotene from δ-carotene is not catalyzed by the lycopene ϵ cyclase of Arabidopsis (Cunningham 1996; U.S. Serial No. 08/624,125) or other known ϵ-cyclases. Therefore, formation of ϵ-cartene and carotenoids derived from it is now made possible with the lettuce lycopene ϵ-cyclase describe herein. Arrows labelled with β indicate reactions synthesize by β-cyclase.

Figure 2 depicts the carotene endgroups which are commonly found in plants.

Figure 3 is a DNA sequence of the romaine lettuce cDNA (SEQ ID NO:1) encoding lycopene epsilon cyclase.

Figure 4 is the predicted amino acid sequence of the romaine lettuce lycopene epsilon cyclase (SEQ ID NO:2).

Figure 5 is a comparison between the predicted amino acid sequences of romaine lettuce (from clone DY4; SEQ ID NO:2) and *Arabidopsis* (from clone y2; SEQ ID NO:3) lycopene epsilon cyclase.

Figure 6 shows the nucleotide and amino acid sequences of the ϵ-cyclase #3 of Adonis palaestina, which also forms bicyclic epsilon carotene (SEQ ID NO: 4 and 5).

Figure 7 Shows a sequence comparison of the Adonis palaestina ϵ-cyclase #3 (SEQ ID NO: 5) compared to the Adonis palaestina ϵ-cyclase #5 (SEQ ID NO: 6), the latter of which adds only a single epsilon ring to lycopene. Five amino acid differences are noted,

which may be targets for site-directed mutagenesis to form the lycopene ϵ-cyclase which adds two ϵ rings to lycopene.

DETAILED DESCRIPTION

Romaine lettuce is one of the rare plant species that produces an abundance of a carotenoid with two epsilon rings (lactucaxanthin). The present inventors have isolated a gene encoding the epsilon cyclase from this plant, and have found that it is similar in sequence to that of *Arabidopsis* (about 65% identity). However, the lettuce enzyme efficiently adds two epsilon rings to lycopene to form the bicyclic epsilon-carotene.

The present invention also relates to methods for transforming known carotenoids into novel or rare products. That is, currently ϵ-carotene (see Figure 1) and γ-carotene can only be isolated in minor amounts. As described below, the enzymes of the invention can be produced and used to transform lycopene to bicyclic ϵ-carotene. With such a product in hand, bulk biosynthesis of other carotenoids derived from the bicyclic epsilon carotene are possible.

The eukaryotic genes in the carotenoid biosynthetic pathway differ from their prokaryotic counterparts in their 5' region. As used herein, the 5' region is the region of eukaryotic DNA which precedes the initiation codon of the counterpart gene in prokaryotic DNA. That is, when the consensus areas of eukaryotic and prokaryotic genes are aligned, the eukaryotic genes contain additional coding sequences upstream of the prokaryotic initiation codon.

The invention also relates to genes encoding lycopene epsilon cyclase which are truncated at the 5' region of the gene. Preferably, such truncated genes are truncated to within 0-50, preferably 0-25, codons of the 5' initiation codon of their prokaryotic counterparts as determined by alignment maps.

In addition to novel enzymes produced by truncating the 5' region of known enzymes, novel enzymes which can participate in the formation of novel carotenoids can be formed by replacing portions of one gene with an analogous sequence from a structurally related gene. The information for adding two epsilon rings can be found in the 3' half of the romaine lettuce gene. Thus, one example of such a hybrid gene construct would include the first half of the romaine lettuce cyclase gene in combination with the second (3') half of

another plant cyclase gene, such as the potato gene or by random of site directed mutagenesis of a mono-ϵ cyclase.

Vectors

The genes encoding the carotenoid enzymes as described above, when cloned into a suitable expression vector, can be used to overexpress these enzymes in a plant expression system or to inhibit the expression of these enzymes. The production or the biochemical activity of the product of *epsilon*-cyclase genes and cDNAs may be reduced or inhibited by a number of different approaches available to those skilled in the art [including but not limited to such methodologies or approaches as anti-sense (e.g., Gray et al (1992)Plant Mol. Biol. 19:69-87), ribozymes (e.g., Wegener et al (1994) Mol. Gen. Genet. 245:465-470), co-suppression (e.g., Fray and Grierson(1993) Plant Mol. Biol. 22:589-602), targeted disruption of the gene (e.g., Schaefer et al. (1997) Plant J. 11:1195-1206), intracellular antibodies (e.g., Rondon and Marasco (1997) Ann. Rev. Microbiol. 51:257-283 or whatever other approaches rely on the knowledge or availability of the gene, cDNA, or polypeptide and/or the sequences of these] to thereby reduce accumulation of carotenoids with *psilon* rings and compounds derived from them.

For example, a vector containing the gene encoding ϵ-cyclase can be used to increase the amount of bicyclic epsilon-carotene in an organism and thereby alter the nutritional value, pharmacology and visual appearance value of the organism. In addition, the transformed organism can be used in the formulation of therapeutic agents, for example in the treatment of cancer (Mayne et al (1996) FASEB J. 10:690-701; Tsushima et al (1995) Biol. Pharm. Bull. 18:227-233, which are both incorporated herein by reference in their entireties).

In a preferred embodiment, the vectors of the present invention contain a DNA encoding an eukaryotic IPP isomerase upstream of a DNA encoding a second eukaryotic carotenoid enzyme. The inventors have discovered that inclusion of an IPP isomerase gene increases the supply of substrate for the carotenoid pathway; thereby enhancing the production of carotenoid endproducts. This is apparent from the much deeper pigmentation in carotenoid-accumulating colonies of *E. coli* which also contain one of the aforementioned IPP isomerase genes when compared to colonies that lack this additional IPP isomerase

gene. Similarly, a vector comprising an IPP isomerase gene can be used to enhance production of secondary metabolites of dimethylallyl pyrophosphate (such as isoprenoids, steroids, carotenoids, etc.).

Alternatively, an anti-sense strand of one of the above genes can be inserted into a vector. For example, the ϵ-cyclase gene can be inserted into a vector and incorporated into the genomic DNA of a host, thereby inhibiting the synthesis of ϵ,β carotenoids (lutein and α-carotene) and enhancing the synthesis of bicyclic epsilon carotenoids.

Suitable vectors according to the present invention comprise a eukaryotic gene encoding an enzyme involved in carotenoid biosynthesis or metabolism and a suitable promoter for the host can be constructed using techniques well known in the art (for example Sambrook et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Suitable vectors for eukaryotic expression in plants are described in Frey et al., Plant J. (1995) 8(5):693 and Misawa et al, 1994; incorporated herein by reference in their entireties.

Suitable vectors for prokaryotic expression include pACYC184, pUC119, and pBR322 (available from New England BioLabs, Bevery, MA), pTrcHis (Invitrogen), Bluescript SK (Stratagene) and pET28 (Novagen) and derivatives thereof.

The vectors of the present invention can additionally contain regulatory elements such as promoters, repressors selectable markers such as antibiotic resistance genes, etc.

Hosts

Host systems according to the present invention can comprise any organism that already produces carotenoids or which has been genetically modified to produce carotenoids.

Organisms which already produce carotenoids include plants, algae, some yeasts, fungi and cyanobacteria and other photosynthetic bacteria. Transformation of these hosts with vectors according to the present invention can be done using standard techniques such as those described in Misawa et al., (1990) supra; Hundle et al., (1993) supra; Hundle et al., (1991) supra; Misawa et al., (1991) supra; Sandmann et al., supra; and Schnurr et al., supra; all incorporated herein by reference in their entireties.

E. coli is an example of one type of bacteria which is suitable as a host for expression of the present enzymes (Cunningham et al, (1996) *The Plant Cell* **8**:1613-1626, which is incorporated herein by reference in its entirety). A vector is used to construct plasmids containing genes encoding the enzymes of the invention, which vector allows it to coexist in *E. coli* with cloning vectors that contain the more common ColE1 origin of replication. The addition of epsilon cyclic end groups to the pink-colored lycopene will result in products that are yellow or orange-yellow in color. Therefore, the functioning of the epsilon lycopene cyclase of the invention may be detected by a change in the color of *E. coli* cultures that accumulate lycopene. Such assays are termed color complementation assays.

Alternatively, transgenic organisms can be constructed which include the DNA sequences of the present invention (Bird et al, 1991; Bramley et al, 1992; Misawa et al, 1994a; Misawa et al, 1994b; Cunningham et al, 1993, all of which are incorporated by reference herein in their entireties). The incorporation of these sequences can allow the controlling of carotenoid biosynthesis, content, or composition in the host cell. These transgenic systems can be constructed to incorporate sequences which allow over-expression of the carotenoid genes of the present invention. Transgenic systems can also be constructed containing antisense expression of the DNA sequences of the present invention. Such antisense expression would result in the accumulation of the substrates of the enzyme encoded by the sense strand.

Appropriate transgenic hosts include lettuce, the natural host, but also other plants such as marigold, tomato, pepper, banana, potato and the like. Essentially any plant is suitable for expressing the present enzyme, but the preferred plants are those which already produce high levels of carotenoids, and those which are normally ingested as foods or used as a source of carotenoid pigments. In particular, plants which bear fruit can be manipulated in such a way as to provide tissue-specific expression in fruit. Marigold is a particularly preferred host, because it can be used as a "bioreactor" for bulk production of carotenoids, and is actually grown commercially as a carotenoid source for chicken feed. For expression in marigold, a promoter can be used which is "flower-specific." Another preferred transgenic plant is tomato, because this plant already produces high levels of lycopene. Indeed, it has been reported that there is a correlation between consuming

tomatoes and decreased incidence of colon cancer (mayne, supra).

<u>A method for screening for eukaryotic genes which encode enzymes involved in carotenoid biosynthesis</u>

The method of the present invention comprises transforming a prokaryotic host with a DNA which may contain a eukaryotic or prokaryotic carotenoid biosynthetic gene; culturing said transformed host to obtain colonies; and screening for colonies exhibiting a different color than colonies of the untransformed host.

Suitable hosts include *E. coli,* cyanobacteria such as *Synechococcus* and *Synechocystis,* alga and plant cells. *E. coli* are preferred.

In a preferred embodiment, the above "color complementation test" can be enhanced by using mutants which are either (1) deficient in at least one carotenoid biosynthetic gene or (2) overexpress at least one carotenoid biosynthetic gene. In either case, such mutants will accumulate carotenoid precursors.

Prokaryotic and eukaryotic genomic and cDNA libraries can be screened in total for the presence of genes of carotenoid biosynthesis, metabolism and degradation. Preferred organisms to be screened include photosynthetic organisms, humans and animals.

E. coli can be transformed with these eukaryotic cDNA libraries using conventional methods such as those described in Sambrook et al, 1989 and according to protocols described by the venders of the cloning vectors.

For example, the cDNA libraries in bacteriophage vectors such as lambdaZAP (Stratagene) or lambdaZIPLOX (Gibco BRL) can be excised en masse and used to transform *E. coli*. Suitable vectors include pACYC184, pUC119, pBR322 (available from New England BioLabs, Bevery, MA). pACYC is preferred.

Transformed *E. coli* can be cultured using conventional techniques. The culture broth preferably contains antibiotics to select and maintain plasmids. Suitable antibiotics include penicillin, ampicillin, chloramphenicol, etc. Culturing is typically conducted at 15-45°C, preferably at room temperature (16-28°C), for 12 hours to 7 days.

Cultures are plated and the plates are screened visually for colonies with a different color than the colonies of the host *E. coli* transformed with the empty vector. For example, *E. coli* transformed with the plasmid, pAC-BETA (described below), produce yellow

colonies that accumulate β-carotene. After transformation with a cDNA library, colonies which contain a different hue than those formed by *E. coli*/pAC-BETA would be expected to contain enzymes which modify the structure or degree of expression of β-carotene. Similar standards can be engineered which overexpress earlier products in carotenoid biosynthesis, such as lycopene, γ-carotene, etc.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE

Isolation of lycopene epsilon cyclase

The lycopene epsilon cyclase was isolated from a romaine lettuce library obtained from Dr. Harry Y. Yamamoto (University of Hawaii, Honolulu) essentially as disclosed in Cunningham et al, 1996, *supra,* and Bugos and Yamamoto (1996) Proc. Natl. Acad. Sci. USA 93:6320-6325, both of which are incorporated herein by reference in their entireties. Functional clones were identified by the color complementation test.

Pigment Analysis

A single colony was used to inoculate 50 ml of LB containing ampicillin and chloramphenicol in a 250-ml flask. Cultures were incubated at 28° C for 36 hours with gentle shaking, and then harvested at 5000 rpm in an SS-34 rotor. The cells were washed once with distilled H_2O and resuspended with 0.5 ml of water. The extraction procedures and HPLC were essentially as described previously (Cunningham et al, 1994).

Organisms and Growth Conditions

E. coli strains TOP10 and TOP10 F' (obtained from Invitrogen Corporation, San Diego, CA) and XL1-Blue (Stratagene) were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37°C in darkness on a platform shaker at 225 cycles per min. Media components were from Difco (yeast extract and tryptone) or Sigma (NaCl). Ampicillin at 150 μg/mL and/or chloramphenicol at 50 μg/mL (both from United States Biochemical Corporation) were used, as appropriate, for selection and maintenance of

plasmids.

Mass Excision and Color Complementation Screening of Romaine Lettuce cDNA Library

A cDNA library of romaine lettuce in lambda ZAPII (Bugos & Yamamoto) was obtained from Henry Yamamoto, as noted above. An aliquot of each library was treated to cause a mass excision of the cDNAs and thereby produce a phagemid library according to the instructions provided by the supplier of the cloning vector (Stratagene; *E. coli* strain XL1-Blue and the helper phage R408 were used). The titre of the excised phagemid was determined and the library was introduced into a lycopene-accumulating strain of *E. coli* TOP10 F' by incubation of the phagemid with the *E. coli* cells for 15 min at 37°C. Cells had been grown overnight at 30°C in LB medium supplemented with 2% (w/v) maltose and 10 mM $MgSO_4$ (final concentration), and harvested in 1.5 ml microfuge tubes at a setting of 3 on an Eppendorf microfuge (5415C) for 10 min. The pellets were resuspended in 10 mM $MgSO_4$ to a volume equal to one-half that of the initial culture volume. Transformants were spread on large (150 mm diameter) LB agar petri plates containing antibiotics to provide for selection of cDNA clones (ampicillin) and maintenance of pAC-LYC (chloramphenicol). Approximately 10,000 colony forming units were spread on each plate. Petri plates were incubated at room temperature for 2 to 7 days to allow maximum color development. Plates were screened visually with the aid of an illuminated 3x magnifier and a low power stage-dissecting microscope for the rare, pale pinkish-yellow to deep-yellow colonies that could be observed in the background of pink colonies. A colony color of yellow or pinkish-yellow was taken as presumptive evidence of a cyclization activity. These yellow colonies were collected with sterile toothpicks and used to inoculate 3ml of LB medium in culture tubes with overnight growth at 37°C and shaking at 225 cycles/min. Cultures were split into two aliquots in microfuge tubes and harvested by centrifugation at a setting of 5 in an Eppendorf 5415C microfuge. After discarding the liquid, one pellet was frozen for later purification of plasmid DNA. To the second pellet was added 1.5 ml EtOH, and the pellet was resuspended by vortex mixing, and extraction was allowed to proceed in the dark for 15-30 min with occasional remixing. Insoluble materials were pelleted by centrifugation at maximum speed for 10 min in a microfuge. Absorption spectra of the supernatant fluids

were recorded from 350-550 nm with a Perkin Elmer lambda six spectrophotometer.

Analysis of isolated clones

Eight of the yellow colonies contained ϵ-carotene indicating that a single gene product catalyzes both cyclizations required to form the two ϵ endgroups of the symmetrical ϵ-carotene from the symmetrical precursor lycopene.

The availability of the romaine lettuce gene encoding the ϵ cyclase enables the directed manipulation of plant and algal species for modification of carotenoid content and composition. Through inactivation of the ϵ cyclase, whether at the gene level by deletion of the gene or by insertional inactivation or by reduction of the amount of enzyme formed (by such as antisense technology), one may increase the formation of β-carotene and other pigments derived from it. Since vitamin A is derived only from carotenoids with β endgroups, an enhancement of the production of β-carotene versus α-carotene may enhance nutritional value of crop plants. Reduction of carotenoids with ϵ endgroups may also be of value in modifying the color properties of crop plants and specific tissues of these plants. Alternatively, where production of α-carotene, or pigments such as lutein that are derived from α-carotene, is desirable, whether for the color properties, nutritional value or other reason, one may overexpress the ϵ cyclase or express it in specific tissues. Wherever agronomic value of a crop is related to pigmentation provided by carotenoid pigments the directed manipulation of expression of the ϵ cyclase gene and/or production of the enzyme may be of commercial value.

The predicted amino acid sequence of the romaine lettuce ϵ cyclase enzyme (SEQ ID NO:2) was determined. A comparison of the amino acid sequences of the ϵ cyclase enzymes of *Arabidopsis thaliana* and romaine lettuce (Figure 5) as predicted by the DNA sequence of the respective genes (Fig. 3 for the ϵ cyclase cDNA sequence), indicates that these two enzymes have many regions of sequence similarity, but they are only about 65% identical overall at the amino acid level.

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Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

<u>Claims:</u>

1. An isolated eukaryotic enzyme which converts lycopene to epsilon, epsilon-carotene.

2. An isolated eukaryotic enzyme of Claim 1 having the amino acid sequence of SEQ ID NO: 2.

3. An isolated DNA sequence comprising a gene encoding the eukaryotic ϵ cyclase of Claim 2.

4. The isolated DNA sequence according to Claim 3, having the nucleic acid sequence of SEQ ID NO: 1.

5. An expression vector comprising the DNA sequence of Claim 3.

6. A host containing the expression vector of Claim 5.

7. The host of Claim 6, wherein said host is *E. coli*.

8. The host of Claim 6, wherein said host is a plant.

9. The host of Claim 8, wherein said host is marigold.

10. The host of Claim 8, wherein said host is tomato.

11. A composition comprising the host of Claim 6.

12. A composition comprising the host of Claim 8.

13. A composition comprising bicyclic epsilon carotene obtained from the host of Claim 6.

14. A composition comprising bicyclic epsilon carotene obtained from the host of Claim 8.

15. A method for treating disease comprising administering to a patient in need thereof, an amount of the composition of Claim 13 sufficient to treat said disease.

16. A method for treating disease comprising administering to a patient in need thereof, an amount of the composition of Claim 14 sufficient to treat said disease.

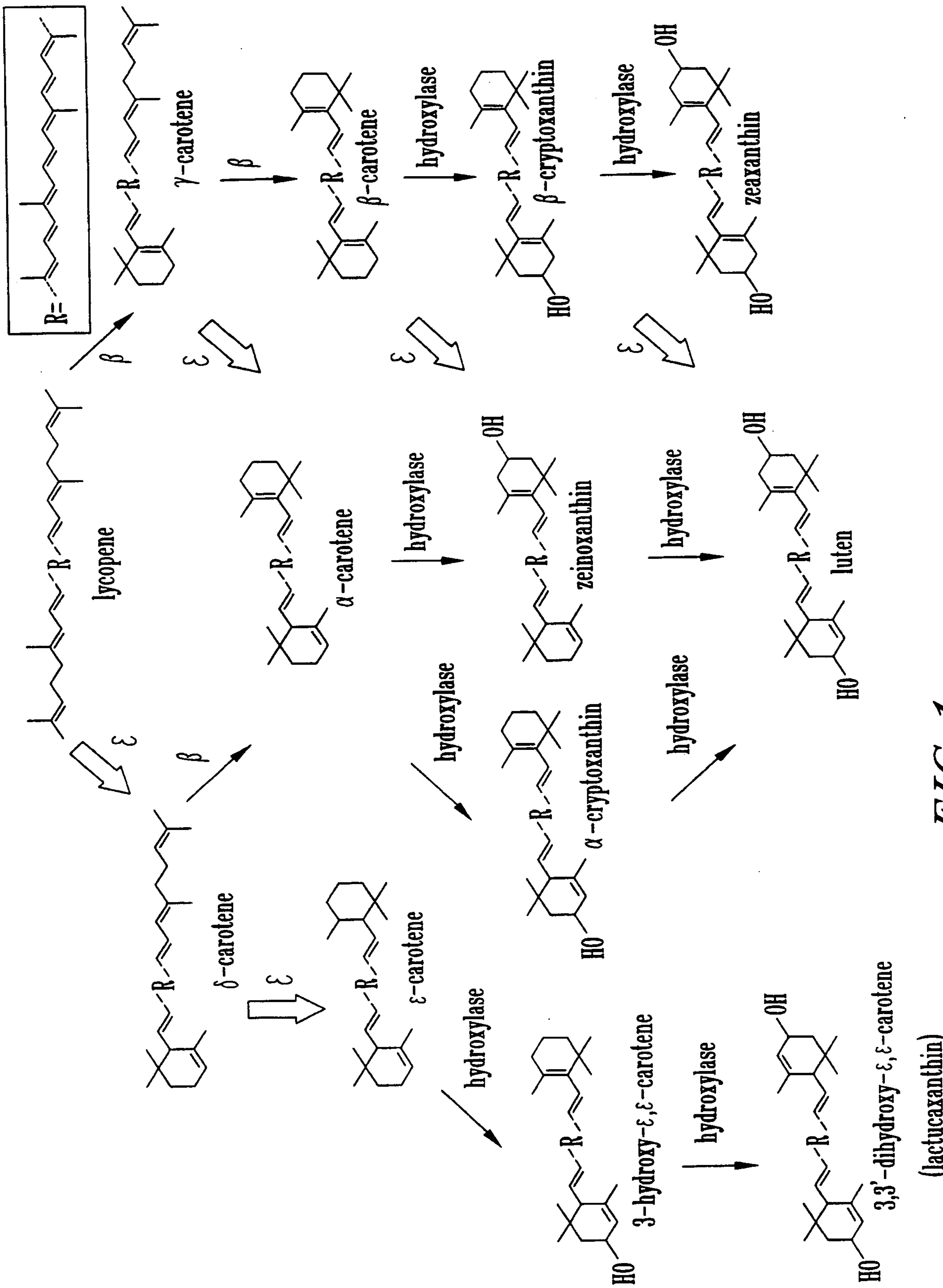
R=
lycopene
γ-carotene
β-carotene
β-cryptoxanthin
zeaxanthin
α-carotene
zeinoxanthin
luten
δ-carotene
ε-carotene
α-cryptoxanthin
3-hydroxy-ε,ε-carotene
3,3'-dihydroxy-ε,ε-carotene
(lactucaxanthin)
hydroxylase
β
ε
HO
OH

FIG. 1

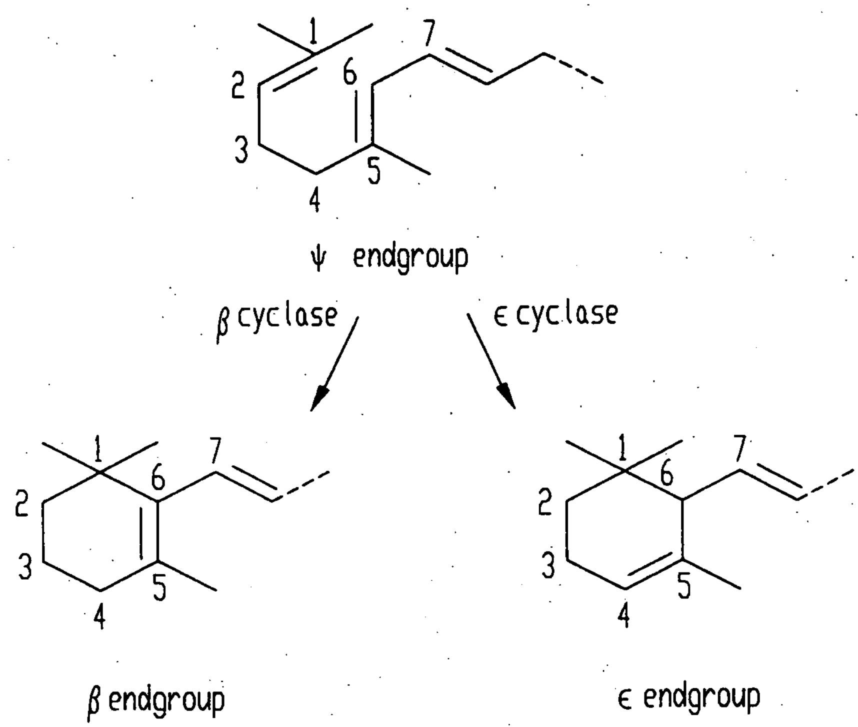

FIG. 2

```
  1 MECFGARNMT ATMAVFTCPR FTDCNIRHKF SLLKQRRFTN LSASSSLRQI
 51 KCSAKSDRCV VDKQGISVAD EEDYVKAGGS ELFFVQMQRT KSMESQSKLS
101 EKLAQIPIGN CILDLVVIGC GPAGLALAAE SAKLGLNVGL IGPDLPFTNN
151 YGVWQDEFIG LGLEGCIEHS WKDTLVYLDD ADPIRIGRAY GRVHRDLLHE
201 ELLRRCVESG VSYLSSKVER ITEAPNGYSL IECEGNITIP CRLATVASGA
251 ASGKFLEYEL GGPRVCVQTA YGIEVEVENN PYDPDLMVFM DYRDFSKHKP
301 ESLEAKYPTF LYVMAMSPTK IFFEETCLAS REAMPFNLLK SKLMSRLKAM
351 GIRITRTYEE EWSYIPVGGS LPNTEQKNLA FGAAASMVHP ATGYSVVRSL
401 SEAPNYAAVI AKILRQDQSK EMISLGKYTN ISKQAWETLW PLERKRQRAF
451 FLFGLSHIVL XDLEGTRTFF RTFFRLPKWM WWGFLGSSLS STDLIIFALY
501 MFVIAPHSLR MELVRHLLSD PTGATMVKAY LTI*
```

FIG. 4

```
1     gaaacaaatg acgtgaaagt tcttcaaaat tgaattaatt gtaatcctga
51    aaacttgatt tgtgatagaa gaatcaatgg agtgctttgg agctcgaaac
101   atgacggcaa caatggcggt ttttacgtgc cctagattca cggactgtaa
151   tatcaggcac aaattttcgt tactgaaaca acgaagattt actaatttat
201   cagcatcgtc ttcgttgcgt caaattaagt gcagcgctaa aagcgaccgt
251   tgtgtagtgg ataaacaagg gatttccgta gcagacgaag aagattatgt
301   gaaggccggt ggatcggagc tgtttttgt tcaaatgcag cggactaagt
351   ccatggaaag ccagtctaaa ctttccgaaa agctagcaca gataccaatt
401   ggaaattgca tacttgatct ggttgtaatc ggttgtggcc ctgctggcct
451   tgctcttgct gcagagtcag ccaaactagg gttgaacgtt ggactcattg
501   gccctgatct tccttttaca aacaattatg gtgtttggca ggatgaattt
551   ataggtcttg gacttgaagg atgcattgaa cattcttgga aagatactct
601   tgtatacctt gatgatgctg atcccatccg cataggtcgt gcatatggca
651   gagttcatcg tgatttactt catgaagagt tgttaagaag gtgtgtggaa
710   tcaggtgttt catatctaag ctccaaagta gaaagaatca ctgaagctcc
751   aaatggctat agtctcattg aatgtgaagg caatatcacc attccatgca
801   ggcttgctac tgttgcatca ggggcagctt cagggaaatt tctggagtat
851   gaacttgggg gtccccgtgt ttgtgtccaa acagcttatg gtatagaggt
901   tgaggttgaa aacaacccct atgatccaga tctaatggtg ttcatggatt
951   atagagactt ctcaaaacat aaaccggaat ctttagaagc aaaatatccg
1001  actttcctct atgtcatggc catgtctcca acaaaaatat tcttcgagga
1051  aacttgttta gcttcaagag aagccatgcc tttcaatctt ctaaagtcca
1101  aactcatgtc acgattaaag gcaatgggta tccgaataac aagaacgtac
1151  gaagaggaat ggtcgtatat ccccgtaggt ggatcgttac ctaatacaga
1201  acaaaagaat ctcgcatttg gtgctgcagc tagtatggtg caccctgcca
1251  cagggtattc agttgttcga tctttgtcag aagctcctaa ttatgcagca
1301  gtcattgcta agattttaag acaagatcaa tctaaagaga tgatttctct
1351  tggaaaatac actaacattt caaaacaagc atgggaaaca ttgtggccac
1401  ttgaaaggaa aagacagcga gccttctttc tattcggact atcacacatc
1451  gtgctaatng atctagaggg aacacgtaca tttttccgta ctttctttcg
1501  tttgcccaaa tggatgtggt ggggattttt ggggtcttct ttatcttcaa
1551  cggatttgat aatatttgcg ctttatatgt ttgtgatagc acctcacagc
1601  ttgagaatgg aactggttag acatctactt tctgatccga caggggcaac
1651  tatggtaaaa gcatatctca ctatatagat ttagattata taaataatac
1701  ccatatcttg catatatata agccttattt atttcttttg taccccccaca
1751  acaacatact cgttaattat atgtttttta
```

FIG. 3

```
  1  MECFGARNMTATMAVFTCPRFTDCNIRHKFSLLKQRRFTNLSASSSLRQI  50
     ||  ||||  | ||| | | .   . | || ..|.  : |:      .
  1  MECVGARNF.AAMAVSTFPSW...SCRRKFPVVKRYSYRNIRFGLCSVRA  46

 51  KCSAKSDRCVVDKQGISVADEEDYVKAGGSELFFVQMQRTKSMESQSKLS  100
           |            |||||:|||||||: |||||. | |: |||
 47  SGGGSSGSESCVAVREDFADEEDFVKAGGSEILFVQMQQNKDMDEQSKLV  96

101  EKLAQIPIGNCILDLVVIGCGPAGLALAAESAKLGLNVGLIGPDLPFTNN  150
     |||  | ||. || |||||||||||||||||||||| ||||||||||||
 97  DKLPPISIGDGALDHVVIGCGPAGLALAAESAKLGLKVGLIGPDLPFTNN  146

151  YGVWQDEFIGLGLEGCIEHSWKDTLVYLDDADPIRIGRAYGRVHRDLLHE  200
     ||||:|||  |||: |||| |::|:|||||  |||||||||| | ||||
147  YGVWEDEFNDLGLQKCIEHVWRETIVYLDDDKPITIGRAYGRVSRRLLHE  196

201  ELLRRCVESGVSYLSSKVERITEAPNGYSLIECEGNITIPCRLATVASGA  250
     |||||||||||||||||: |||| .|  |: |: |  |||||||||| |
197  ELLRRCVESGVSYLSSKVDSITEASDGLRLVACDDNNVIPCRLATVASGA  246

251  ASGKFLEYELGGPRVCVQTAYGIEVEVENNPYDPDLMVFMDYRDFSKHKP  300
     || |:||.|||||||||||||:||||||.||||| ||||||||:.
247  ASGKLLQYEVGGPRVCVQTAYGVEVEVENSPYDPDQMVFMDYRDYTNEKT  296

301  ESLEAKYPTFLYVMAMSPTKIFFEETCLASREAMPFNLLKSKLMSRLKAM  350
      ||||.|||||| | |.|.::|| |||| |:: |||.|||.|||     :
297  RSLEAEYPTFLYAMPMTKSRLFFEETCLASKDVMPFDLLKTKLMLRLSTL  346

351  GIRITRTYEEEWSYIPVGGSLPNTEQKNLAFGAAASMVHPATGYSVVRSL  400
     |||| :||||||||||||||||||||||||||||||||||||||||||||
347  GIRILKTYEEEWSYIPVGGSLPNTEQKNLAFGAAASMVHPATGYSVVRSL  396

401  SEAPNYAAVIAKILRQDQSKEMISLGKYTNISKQAWETLWPLERKRQRAT  450
     |||| ||.|||.|||::  .|:. |     |||:|||:| || ||||||
397  SEAPKYASVIAEILREETTKQINS.....NISRQAWDTLWPPERKRQRAF  446

451  FLFGLSHIVLMDLEGTRTFFRTFFRLPKWMWWGFLGSSLSSTDLIIFALY  500
     |||. || |      .||||||||||||| |||||. .||||::||||
442  FLFGLALIVQFDTEGIRSFFRTFFRLPKWMWQGFLGSTLTSGDLVLFALY  496

501  MFVIAPHSLRMELVRHLLSDPTGATMYKAYLTI*  534
     |.|..||    :  :          :      :
492  MFVISPNNLRKGLINHLISDPTGATMIKTYLKV*  524
```

FIG. 5

Adonis palaestina ε-cyclase cDNA #3 Length 1848

1	gagagaaaaa	gagtgttata	ttaatgttac	tgtcgcattc	ttgcaacaca
51	tattcagact	ccattttctt	gttttctctt	caaaacaaca	aactaatgtg
101	acggagtatc	tagctatgga	actacttggt	gttcgcaacc	tcatctcttc
151	ttgccctgtc	tggacttttg	gaacaagaaa	ccttagtagt	tcaaaactag
201	cttataacat	acatcgatat	ggttcttctt	gtagagtaga	ttttcaagtg
251	agggctgatg	gtggaagcgg	gagtagaact	tctgttgctt	ataaagaggg
301	ttttgtggac	gaggaggatt	ttatcaaagc	tggtggttct	gagcttttgt
351	ttgtccaaat	gcagcaaaca	aagtctatgg	agaaacaggc	caagctcgcc
401	gataagttgc	caccaatacc	tttcggagaa	tctgtgatgg	acttggttgt
451	aataggttgt	ggacctgctg	gtctttcact	ggctgcagaa	gctgctaagc
501	taggcttgaa	agttggcctt	attggtcctg	atcttccttt	tacaaataat
551	tatggtgtgt	gggaagacga	gttcaaagat	cttggacttg	aacgttgtat
601	cgagcatgct	tggaaggaca	ccatcgtata	tcttgacaat	gatgctcctg
651	tccttattgg	tcgtgcatat	ggacgagtta	gccggcattt	gctgcatgaa
701	gagttgctga	aaaggtgtgt	cgagtcaggt	gtatcatatc	tgaattctaa
751	agtggaaagg	atcactgaag	ctggtgatgg	ccatagtctt	gtagtttgtg
801	aaaacgacat	ctttatccct	tgcaggcttg	ctactgttgc	atctggagca
851	gcttcaggga	aacttttgga	gtatgaagta	ggtggccctc	gtgtttgtgt
901	ccaaactgct	tatggtgtgg	aggttgaggt	ggagaacaat	ccatacgatc
951	ccaacttaat	ggtatttatg	gactacagag	actatatgca	acagaaatta
1001	cagtgctcgg	aagaagaata	tccaacattt	ctctatgtca	tgcccatgtc
1051	gccaacaaga	cttttttttg	aggaaacctg	tttggcctca	aaagatgcca
1101	tgcctttcga	tctactgaag	agaaaactaa	tgtcacgatt	gaagactctg
1151	ggtatccaag	ttacaaaaat	ttatgaagag	gaatggtctt	atattcctgt
1201	tgggggttct	ttaccaaaca	cagagcaaaa	gaacctagca	tttggtgctg
1251	cagcaagcat	ggtgcatcca	gcaacaggct	attcggttgt	acgatcacta
1301	tcagaagctc	caaaatatgc	ttctgtaatt	gcaaagattt	tgaagcaaga
1351	taactctgca	tatgtggttt	ctggacaaag	cagtgcagta	aacatttcaa
1401	tgcaagcatg	gagcagtctt	tggccaaagg	agcgaaaacg	tcaaagagca
1451	ttctttcttt	tcgggttaga	gcttattgtg	cagctagata	ttgaagcaac
1501	cagaacgttc	tttagaacct	tcttccgctt	gccaacttgg	atgtggtggg
1551	gtttccttgg	gtcttcacta	tcatctttcg	atcttgtatt	gttttccatg
1601	tacatgtttg	ttttggcccc	gaacagcatg	aggatgtcac	ttgtgagaca
1651	tttgctttca	gatccttctg	gtgcagttat	ggttaaagct	tacctcgaaa
1701	ggtaatctgt	tttatgaaac	tatagtgtct	cattaaataa	atgaggatcc
1751	ttcgtatatg	tatatgatca	tctctatgta	tatcctatat	tctaatctca
1801	taaagtaatc	gaaaattcat	tgatagaaaa	aaaaaaaaaa	aaaaaaaa

FIG. 6A

Adonis palaestina ε-cyclase #3 predicted polypeptide
TRANSLATE from: 116 to: 1705 Length : 529 amino acids

```
  1 MELLGVRNLI SSCPVWTFGT RNLSSSKLAY NIHRYGSSCR VDFQVRADGG
 51 SGSRTSVAYK EGFVDEEDFI KAGGSELLFV QMQQTKSMEK QAKLADKLPP
101 IPFGESVMDL VVIGCGPAGL SLAAEAAKLG LKVGLIGPDL PFTNNYGVWE
151 DEFKDLGLER CIEHAWKDTI VYLDNDAPVL IGRAYGRVSR HLLHEELLKR
201 CVESGVSYLN SKVERITEAG DGHSLVVCEN DIFIPCRLAT VASGAASGKL
251 LEYEVGGPRV CVQTAYGVEV EVENNPYDPN LMVFMDYRDY MQQKLQCSEE
301 EYPTFLYVMP MSPTRLFFEE TCLASKDAMP FDLLKRKLMS RLKTLGIQVT
351 KIYEEEWSYI PVGGSLPNTE QKNLAFGAAA SMVHPATGYS VVRSLSEAPK
401 YASVIAKILK QDNSAYVVSG QSSAVNISMQ AWSSLWPKER KRQRAFFLFG
451 LELIVQLDIE ATRTFFRTFF RLPTWMWWGF LGSSLSSFDL VLFSMYMFVL
501 APNSMRMSLV RHLLSDPSGA VMVKAYLER*
```

FIG. 6B

```
GAP program of Genetics Computer Group
blosum 62.cmp
         Gap Weight:      12          Average Match:     2.912
      Length Weight:       4       Average Mismatch:    -2.003
            Quality:    2728                 Length:       530
              Ratio:   5.147                   Gaps:         0
Percent Similarity:   99.623       Percent Identity:    99.057
Match display thresholds for the alignment (s): | = IDENTITY : =2 . =1
Adonis palaestina ∈-cyclase #3 x Adonis palaestina ∈-cyclase #5


         .         .         .         .         .
  1  MELLGVRNLISSCPVWTFGTRNLSSSKLAYNIHRYGSSCRVDFQVRADGG   50
     ||||||||||||||||||||||||||||||||||||||||||||||||||
  1  MELLGVRNLISSCPVWTFGTRNLSSSKLAYNIHRYGSSCRVDFQVRADGG   50

         .         .         .         .         .
 51  SGSRTSVAYKEGFVDEEDFIKAGGSELLFVQMQQTKSMEKQAKLADKLPP  100
     ||||.|||||||||||||||||||||||||||||||||||||||||||||
 51  SGSRSSVAYKEGFVDEEDFIKAGGSELLFVQMQQTKSMEKQAKLADKLPP  100

         .         .         .         .         .
101  IPFGESVMDLVVIGCGPAGLSLAAEAAKLGLKVGLIGPDLPFTNNYGVWE  150
     ||||||||||||||||||||||||||||||||||||||||||||||||||
101  IPFGESVMDLVVIGCGPAGLSLAAEAAKLGLKVGLIGPDLPFTNNYGVWE  150

         .         .         .         .         .
151  DEFKDLGLERCIEHAWKDTIVYLDNDAPVLIGRAYGRVSRHLLHEELLKR  200
     ||||||||||||||||||||||||||||||||||||||||||||||||||
151  DEFKDLGLERCIEHAWKDTIVYLDNDAPVLIGRAYGRVSRHLLHEELLKR  200

         .         .         .         .         .
201  CVESGVSYLNSKVERITEAGDGHSLVVCENDIFIPCRLATVASGAASGKL  250
     ||||||||||.|||||||||||||||||||||:|||||||||||||||||
201  CVESGVSYLDSKVERITEAGDGHSLVVCENEIFIPCRLATVASGAASGKL  250

         .         .         .         .         .
251  LEYEVGGPRVCVQTAYGVEVEVENNPYDPNLMVFMDYRDYMQQKLQCSEE  300
     ||||||||||||||||||||||||||||||||||||||||||||||||||
251  LEYEVGGPRVCVQTAYGVEVEVENNPYDPNLMVFMDYRDYMQQKLQCSEE  300
```

FIG. 7A

```
301 EYPTFLYVMPMSPTRLFFEETCLASKDAMPFDLLKRKLMSRLKTLGIQVT 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 EYPTFLYVMPMSPTRLFFEETCLASKDAMPFDLLKRKLMSRLKTLGIQVT 350

351 KIYEEEWSYIPVGGSLPNTEQKNLAFGAAASMVHPATGYSVVRSLSEAPK 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 KVYEEEWSYIPVGGSLPNTEQKNLAFGAAASMVHPATGYSVVRSLSEAPK 400

401 YASVIAKILKQDNSAYVVSGQSSAVNISMQAWSSLWPKERKRQRAFFLFG 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 YASVIAKILKQDNSAYVVSGQSSAVNISMQAWSSLWPKERKRQRAFFLFG 450

451 LELIVQLDIEATRTFFRTFFRLPTWMWWGFLGSSLSSFDLVLFSMYMFVL 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 LELIVQLDIEATRTFFRTFFRLPTWMWWGFLGSSLSSFDLVLFSMYMFVL 500

501 APNSMRMSLVRHLLSDPSGAVMVKAYLER* 530
    ||||||||||||||||||||||| |||||
501 APNSMRMSLVRHLLSDPSGAVMVRAYLER* 530
```

FIG. 7B

SEQUENCE LISTING

<110> CUNNINGHAM JR., FRANCIS X.
SUN, ZAIREN

<120> GENES ENCODING EPSILON LYCOPENE CYCLASE AND METHOD FOR
PRODUCING BICYCLIC EPSILON CAROTENE

<130> 2747-0084-27 CIP

<140> 09/084,222
<141> 1998-05-26

<150> 08/937,155
<151> 1997-09-25

<150> 08/624,125
<151> 1996-03-29

<160> 6

<170> PatentIn Ver. 2.0

<210> 1
<211> 1780
<212> DNA
<213> romaine lettuce
<223> n is an unspecified nucleotide

<400> 1
gaaacaaatg acgtgaaagt tcttcaaaat tgaattaatt gtaatcctga aaacttgatt 60
tgtgatagaa gaatcaatgg agtgctttgg agctcgaaac atgacggcaa caatggcggt 120
ttttacgtgc cctagattca cggactgtaa tatcaggcac aaattttcgt tactgaaaca 180
acgaagattt actaatttat cagcatcgtc ttcgttgcgt caaattaagt gcagcgctaa 240
aagcgaccgt tgtgtagtgg ataaacaagg gatttccgta gcagacgaag aagattatgt 300
gaaggccggt ggatcggagc tgttttttgt tcaaatgcag cggactaagt ccatggaaag 360
ccagtctaaa ctttccgaaa agctagcaca gataccaatt ggaaattgca tacttgatct 420
ggttgtaatc ggttgtggcc ctgctggcct tgctcttgct gcagagtcag ccaaactagg 480
gttgaacgtt ggactcattg gccctgatct tcctttaca aacaattatg gtgtttggca 540
ggatgaattt ataggtcttg gacttgaagg atgcattgaa cattcttgga aagatactct 600
tgtatacctt gatgatgctg atcccatccg cataggtcgt gcatatggca gagttcatcg 660
tgatttactt catgaagagt tgttaagaag gtgtgtggaa tcaggtgttt catatctaag 720
ctcccaaagta gaaagaatca ctgaagctcc aaatggctat agtctcattg aatgtgaagg 780
caatatcacc attccatgca ggcttgctac tgttgcatca ggggcagctt cagggaaatt 840
tctggagtat gaacttgggg gtccccgtgt ttgtgtccaa acagcttatg gtatagaggt 900
tgaggttgaa aacaacccct atgatccaga tctaatggtg ttcatggatt atagagactt 960
ctcaaaacat aaaccggaat ctttagaagc aaaatatccg actttcctct atgtcatggc 1020
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Ser Ser Leu Trp Pro Lys Glu Arg Lys Arg Gln Arg Ala Phe Phe Thr
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Leu Phe Gly Leu Glu Leu Ile Val Gln Leu Asp Ile Glu Ala Thr Arg
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Thr Phe Phe Arg Thr Phe Phe Arg Leu Pro Thr Trp Met Trp Trp Gly
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His Leu Leu Ser Asp Pro Ser Gly Ala Val Met Val Arg Ala Tyr Leu
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10461

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07C 13/00; C12N 9/00, 15/00, 5/00; A01N 27/00
US CL :585/23; 435/183, 320.1, 325; 514/763
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 585/23; 435/183, 320.1, 325; 514/763

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS, SCISEARCH, LIFESCI, BIOTECHDS, NTIS, EMBASE, BIOSIS, HCAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - A	CUNNINGHAM et al. Functional analysis of the beta and epsilon Lycopene Cyclase enzymes of Arabidopsis reveals a mechanism for control of Cyclic Caroteniod formation. The Plant Cell. September 1996, Vol.8, pages 1613-1626, see the entire article.	1 ---- 2-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 02 AUGUST 1999	Date of mailing of the international search report 10 SEP 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D. Lawrence for MARYAM MONSHIPOURI Telephone No. (703) 308-0196